'Touchdown' PCR to circumvent spurious priming during gene amplification

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A frequently encountered problem in PCR amplification of target gene sequences, especially from complex genomes, is the appearance of spurious smaller bands in the product spectrum. This is usually interpreted to be due to mispriming by one or both of the oligonucleotide amplimers internal or external to the target template. These alternative products frequently dominate the reaction products and are presumably due to the repetitive stochastic advantage that shorter misprimed products enjoy over the longer correct product during reaction cycling. This problem is therefore compounded over increasing numbers of cycles and is thus more likely to occur where target template is present only in small amounts. Adjustments to the [Mg^{++}] or increasing the annealing temperature of the PCR may solve this problem presuming that the spurious interactions are sufficiently less stable than the specific (correct) ones due to degrees of sequence mismatch. Such empirical determinations are time-consuming. We devised a simple solution taking advantage of the exponential nature of PCR reactions, and which begins at or above, rather than below, the expected annealing temperature. The annealing temperature of the reaction is decreased 1°C (in this example) every second cycle from 65°C to a 'touchdown' at 55°C, at which temperature 10 cycles are carried out. Any difference in Tm between the correct and incorrect annealings will give an advantage of 2-fold per cycle, or 4-fold per °C, to the correct product, all else being equal. Hence, a 5°C difference would give a 4^5 (i.e., 1024)-fold advantage.

We used this method in a case where we had designed primers to amplify only the cDNA sequences coding for leukemia inhibitory factor (LIF). LIF is a glycoprotein capable both of inducing the differentiation of M1 myeloid leukemia cells (1) and of inhibiting the differentiation of murine embryonic stem cells (2, 3). PCR from 100 ng of chromosomal DNA using primers at the 5′ and 3′ ends of the protein coding sequences, at an annealing temperature of 55°C, gave the expected ~1100 bp product (including intronic sequences), indicating that the primers were finding the appropriate target (Figure 1, lane 2). PCR amplification from cDNA under standard conditions (4), however, yielded prominent small bands, largely between 100 and 200 bp in size. This was only exacerbated by the traditional processive approach of incrementally raising the annealing temperature every ten cycles during the PCR, for instance from 45°C to 50°C to 55°C (Figure 1, lane 3). Presumably if we had persevered beyond 55°C and varied the increment we would eventually have found a discriminatory annealing temperature which favoured the correct product. Using the touchdown strategy the imbalance between correct and spurious annealing was automatically redressed, and allowed amplification of sufficient correct length LIF cDNA sequence (540 bp) for cloning (Figure 1, lane 4). (A 250 bp secondary band also appeared, presumably because it also gained a competitive advantage over the smaller products during the touchdown PCR. Scanning of the LIF cDNA sequence (5) showed this band to be probably due to internal priming of the 3′ amplimer at a 14/20 bp match to the template 384–403 bp downstream from the start codon.) This approach should be applicable to a wide variety of such situations, conveniently by-passing spurious amplifications without lengthy optimization procedures. Even in cases where an appropriate discriminatory temperature has been empirically determined, the touchdown approach could also help avoid secondary problems, such as an inconsistency of well temperatures within or between thermal cycling machines.

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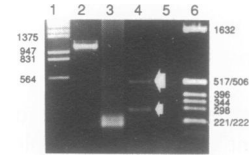

Figure 1. Lane 1: size standards (Lambda DNA digested by *Hind*III and *Eco*RI). Lane 2: Amplification from amplimers BR1 (5′-CCTCTTCCCATCACCCC-TGT-3′) and BR2 (5′-CTAGAAGGCCTGGACCACCA-3′) using 100 ng mouse chromosomal DNA at a constant annealing temperature (55°C). Lane 3: PCR gradually raising the annealing temperature from 45°C to 55°C. Lane 4: 'Touchdown' PCR. Lane 5: No reverse transcriptase control PCR from the same experiment as lane 4. Lane 6: Size standards (pBR322 digested with *Hin*fI). 5 μl of each 100 μl PCR was electrophoresed per track on a 1.6% agarose gel.

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